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Heavy metal availability and impact on activity of soil microorganisms along a Cu/Zn contamination gradient

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Abstract

All the regulations that define a maximum concentration of metals in the receiving soil are based on total soil metal concentration. However, the potential toxicity of a heavy metal in the soil depends on its speciation and availability. We studied the effects of heavy metal speciation and availability on soil microorganism activities along a Cu/Zn contamination gradient. Microbial biomass and enzyme activity of soil contaminated with both Cu and Zn were investigated. The results showed that microbial biomass was negatively affected by the elevated metal levels. The microbial biomass-C (C_{mic})/organic C (C_{org}) ratio was closely correlated to heavy metal stress. There were negative correlations between soil microbial biomass, phosphatase activity and NH_4NO_3 extractable heavy metals. The soil microorganism activity could be predicted using empirical models with the availability of Cu and Zn. We observed that 72% of the variation in phosphatase activity could be explained by the NH_4NO_3-extractable and total heavy metal concentration. By considering different monitoring approaches and different viewpoints, this set of methods applied in this study seemed sensitive to site differences and contributed to a better understanding of the effects of heavy metals on the size and activity of microorganisms in soils. The data presented demonstrate the relationship between heavy metals availability and heavy metal toxicity to soil microorganism along a contamination gradient.

Key words: heavy metals; availability; speciation; activity; soil microorganism

Introduction

The spread of heavy metals in the terrestrial environment is largely attributed to the disposal of waste products (Kovalchuk *et al*., 2001; Lasat, 2002). Many reports have shown that short-term or long-term exposure to heavy metals results in the reduction of microbial diversity and activities in soil (Gadd, 1993; Brookes, 1995; Elsgaard *et al*., 2001; Sandaa *et al*., 2001; Akmal *et al*., Akmal *et al*., 2005). Diversity and activity of microbial communities are important indeces of soil quality. Soil microbes play a significant role in recycling of plant nutrients, maintenance detoxification of noxious chemicals, and the control of plant pests and plant growth (Brookes, 1995; Giller *et al*., 1998). Alterations in the activity of microorganisms have often been proposed as a sensitive indicator of anthropogenic effects on soil ecology (Shi *et al*., 2002; Renella *et al*., 2005).

Copper (Cu) and Zinc (Zn) are essential elements. Elevated levels of Cu and Zn in soil adversely affect microbially mediated soil processes. The European Union Council's directive limits for concentrations of heavy metals in arable soils indicate limits as 140 mg/kg for Cu, 300 mg/kg for Zn (Council of the European Communities, 1986). Soil microbial biomass, which plays an important role in nutrient cycling and ecosystem sustainability, has been found to be sensitive to increased heavy metal concentrations in soils (Brookes, 1995; Filip, 2002). In most studies involving soil microorganisms or plants in soils, toxicity of heavy metals has been related to their total concentrations in soil (Sitaula *et al*., 1999; Huang and Shindob, 2000). Existing legislation or guidelines on heavy metals in soils are also based on total concentrations (Council of the European Communities, 1986). However, previous studies showed that every bio-indicator does not respond consistently to heavy metal contamination in the soil because soil systems are usually more complex and usually have large spatial variation, which do not reflect heavy metals availability. When assessing the toxicity of contaminated soil, both heavy metal concentration and availability must first be determined.

For derivation of soil quality criteria, it is important that toxicities to soil microorganisms are linked directly to heavy metals availability (Kot and Namiesnèik, 2000). So far, the information linking heavy metals availability

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and toxicological responses in soils is very limited. In this study, the relationships between microbial features and Cu/Zn availability were investigated to clarify the availability of Cu and Zn, and its effects on microorganisms in soils from a contaminated site in the south of China. Water and NH_4NO_3 tests were used for multi-element extraction of micronutrients and has been used for assessing the bioavailability of non-essential trace metals (Sauvé *et al*., 2000; Gummuluru *et al*., 2002). Soil microbiological and biochemical biochemical properties (biomass C, phosphatase activities) were measured to determine the effects of heavy metals on microbial activities.

1 Materials and methods

1.1 Study site

The area selected is the downwind of a copper-zinc smelter, which was started in 1985 in Zhujiawu County, Zhejiang Province, China. The soil was contaminated by heavy metals, mainly Cu and Zn. According to USDA soil taxonomy (United States Department of Agriculture), the Alluvial sandy loam, paddy soil that developed on the river alluvium is fluvaquents. This area was formerly used to cultivate cereal grass. After 1990s the site has not been used for cultivation due to the reduction of crop output and contamination of the crop by heavy metals. The site was covered with the plants tolerant to heavy metals at the time of sampling.

1.2 Soil samples

The soils were sampled along a gradient of pollution of Cu and Zn. The samples sites included A1–A7 (50–150 m from the smelter, numbered from 1 to 7), B1–B7 (150–250 m), C1–C7 (250–400 m), D1–D7 (400–500 m), and E1–E7 (500–600 m, the farthest from the smelter). Each sample included 6 cores of 2 cm diameter and 0–15 cm depth. Thirty five samples were taken from the area. Field moist soils were sieved through a mesh < 2 mm and large pieces of plant material, stones and soil animals were removed. Part of samples was kept moist in the dark at 4°C to determine the heavy metals in the soil solution and assess soil enzyme activity and microbial biomass. The remaining soil was air-dried at ambient temperature, crushed and sieved through 0.2 mm mesh to analyze pH, organic matter and total metals content.

1.3 Soil analysis

Soil pH was measured with a pH meter (PHS-3C) using a 1:2.5 soil-to-water solution. Organic C was determined by dichromate digestion (Lu, 1999). Total contents of Cu/Zn in the soil were analyzed by digesting 100 mg of soil in a mixture of HF-$HClO_4$-HNO_3 (Lu, 1999) and determining metal concentrations in the digester using a flame atomic absorption spectrophotometer with graphite (GFAAS) (Thermo Element MKII-M6). Available Cu and Zn in the soil were extracted with NH_4NO_3 (1:2.5, w/v). The soil suspensions were centrifuged at 4000 ×*g* for 10 min and filtered. Cu and Zn content in each filtrate was determined by the FAAS. All samples were analyzed in triplicate.

1.4 Cu and Zn fractionation

Particulate-bound Cu and Zn fractions in the soils were determined following the sequential extraction scheme of Table 1 (Gummuluru *et al*., 2002; Huang *et al*., 2004). The sequential extraction was carried out in triplicate with 1 g of soil (< 0.2 mm) in 50-ml polypropylene centrifuge tubes. Centrifuge tubes were used to facilitate centrifuge washing of the soil after each extraction, thus minimizing any loss of the solids. After each successive extraction, the supernatant was removed by centrifugation for 10 min at 8000 ×*g*. The residue was washed once with 5 ml of deionized distilled water by centrifugation at 10000 ×*g* for 10 min, and the supernatants were combined and made up to a known volume.

1.5 Saturation water extract

Sufficient amounts of deionized water were added to 50 g of the fresh soil in duplicate to bring the soil to saturation. The soil was mixed thoroughly to form slurry and equilibrated for 24 h in a 250-ml plastic container. Soil solutions were obtained from the slurry by filtration under vacuum using filter paper. The solutions were centrifuged at 12000 ×*g* for 30 min and passed through 0.25 μm Millipore filters (Sauvé *et al*., 2000). The concentration of metals was determined by the GFAAS.

1.6 Microbial biomass carbon and soil enzyme activity

The fumigation-extraction method described by Vance *et al*. (1987) was used, and the total carbon contents of the 0.5 mol/L K_2SO_4 extracts were measured with an automated TOC Analyser (Shimadzu, TOC-500, Japan). Biomass C (B_C) was calculated from: B_C=2.22, E_C where E_C=(C extracted from fumigated soil)–(C extracted from non-fumigated soil). Soil phosphatase activity was measured spectrophotometrically by the disodium phenyl phosphate

Table 1 Multi-step selective sequential extraction scheme for particulate-bound Cu and Zn fractions

Step	Fractions	Reagent	Shaking time and temperature
1	Exchangeable (EXC)	8 ml of 1 mol/L NH_4NO_3 (pH 7)	2 h at 25°C
2	Bound to carbonates (CAR)	8 ml aliquot of NaAc (pH 5.0 with HOAc)	5 h at 25°C
3	Amorphous iron-manganese oxides (OXI)	20 ml of 0.04 mol/L hydroxylamine hydrochloride in 25% acetic acid (pH 2 with HNO_3)	6 h at 25°C
4	Organic-bound (ORG)	5 ml of 30% H_2O_2 (pH 2), 3 ml of 0.02 mol/L HNO_3 and	2 h at 85°C
		3 ml of 30% H_2O_2 (pH 2)	2 h at 85°C
		Cool, add 20 ml of a mixture of 3.2 mol/L NH_4Ac and 20% HNO_3	30 min at 25°C
5	Residual (RES)	The residual fraction was derived by subtracting these four fractions from the total	

method of Li (1996).

1.7 Statistical analysis

All data were analyzed using Microsoft Excel, and SPSS 11.0. A probability level of 0.05 was considered to be statistically significant. Soil microbial biomass and enzyme activity were log-transformed before analysis to stabilize the variance. Correlation analyses were performed to determine relationships among heavy metals, soil microbial biomass, enzyme activity.

2 Results

2.1 Heavy metal content in soil and other soil factors

In the last 20 years, Cu and Zn have been the dominant sources of pollution in this area. Selected properties of soil solid phase are shown in Table 2. The soils varied greatly in heavy metal concentrations due to their distances from the smelter. Soil pH varied from 7.64 to 8.27, decreasing along the Cu/Zn contamination gradient. The range of soil organic matter was from 30.1–40.7 g/kg. There was a large variation in the concentration of total Cu (121–833 mg/kg) and Zn (914–4700 mg/kg). The total concentrations of Cu, Zn were higher in sites close to the smelter and decreased with the distance from the smelter. The highest concentration of Cu was found in area A but Zn in area B. No significant correlation was found between soil total heavy metal concentration and soil organic C.

2.2 Heavy metal availability

The concentrations of soluble Cu and Zn in the pore water samples varied from 0.12 to 5.12 mg/L and 0.13 to 3.20 mg/L, respectively (Fig.1). Cu and Zn concentrations in the soil solution decreased along the distance from the smelter. The concentrations of NH_4NO_3-extractable heavy metals followed a similar trend. The differences in Cu and Zn concentrations in soil solution from A1 to E7 were consistent with the NH_4NO_3-extracted Cu and Zn concentrations, which showed a maximum of 12.46 mg/kg for Cu in A1 and 21.74 mg/kg for Zn in A4. There was significant relationship between pH and NH_4NO_3 extractable, total, or water soluble Cu and Zn. NH_4NO_3 extractable Zn had the best correlation (0.70) with soil pH ($P<0.01$) (Table 3).

Table 2 Select properties of soil samples collected along heavy metals pollution gradient

Site		Total Zn (mg/kg)	Total Cu (mg/kg)	Organic matter (g/kg)	pH[b]
Distance[a]	Number				
50–150 m	A1	2740	833	36.4	7.78
	A2	3839	697	32.6	7.71
	A3	3603	668	36.1	7.96
	A4	3278	586	36.9	7.64
	A5	2510	506	37.8	7.65
	A6	2282	485	35.9	7.79
	A7	2269	421	36.3	7.74
150–250 m	B1	2832	406	36.5	7.91
	B2	2538	479	36.8	7.99
	B3	2011	400	37.0	8.04
	B4	2290	370	40.3	7.98
	B5	1361	204	35.1	7.72
	B6	3837	375	36.8	7.93
	B7	4700	360	37.3	7.80
250–400 m	C1	3905	286	35.2	7.85
	C2	1810	262	37.7	7.97
	C3	2741	249	32.9	8.17
	C4	2597	248	36.1	7.99
	C5	3552	245	33.6	7.93
	C6	3275	221	35.8	7.78
	C7	3113	208	36.4	7.95
400–500 m	D1	1404	192	39.0	8.06
	D2	1310	188	38.0	8.1
	D3	1296	169	36.1	8.21
	D4	1128	168	38.0	8.27
	D5	1099	166	38.6	8.14
	D6	1319	166	39.1	8.09
	D7	1158	161	38.4	8.11
500–600 m	E1	1453	187	37.0	8.07
	E2	1374	169	36.6	7.99
	E3	1245	163	37.0	8.03
	E4	1266	147	40.7	8.18
	E5	914	133	36.6	8.19
	E6	1162	121	35.8	8.08
	E7	1114	157	30.1	8.14

[a] Distance from the smelter; [b] (H_2O : Soil=2.5 : 1).

2.3 Fractionation of Cu and Zn in the soils

The metal fractions consist of (1) exchangeable (EXC); (2) bound to carbonates (CAR); (3) amorphous iron-manganese oxides (OXI); (4) organic-bound (ORG) and (5) residual (RES), were extracted from the bulk soil samples (Table 1). The amounts of each metal in each fraction are presented in Fig.2. The mean concentrations of

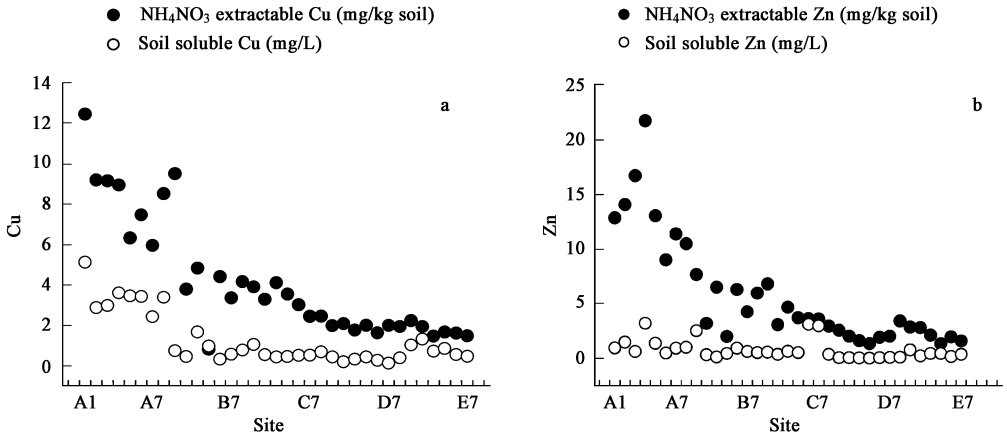

Fig. 1 Distribution of NH_4NO_3-extractable and water soluble heavy metals along the copper-zinc smelter. (a) Cu; (b) Zn.

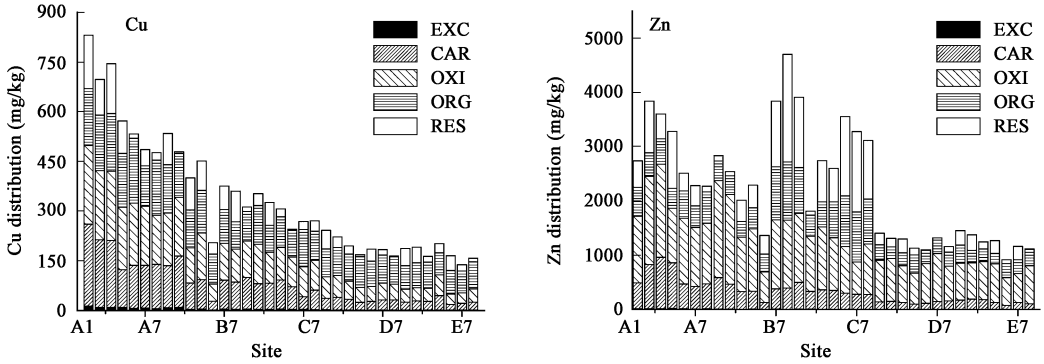

Fig. 2 Distribution of the five Cu and Zn fractionations in the soil along a contamination gradient.

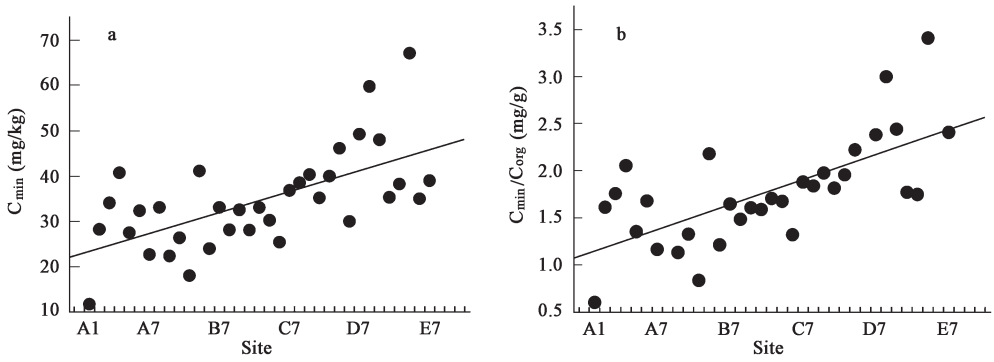

Fig. 3 Microbial biomass C (C_{min}) and their ratios to organic C (C_{min}/C_{org}) in the heavy metal contaminated soils.

Table 3 Correlation coefficients (*r*) among microbial parameters and soil heavy metals, pH

	Cu			Zn		
	Soluble [a]	NH_4NO_3 [b]	Total [c]	Soluble	NH_4NO_3	Total
pH	–0.65**	–0.59**	–0.64**	–0.56**	–0.70**	–0.64**
lg (C_{mic})[1]	–0.43*	–0.60**	–0.64**	–0.22	–0.38*	–0.48**
lg (P)[2]	–0.47**	–0.85**	–0.82**	–0.68**	–0.76**	–0.61**
C_{mic}/C_{org}	–0.37*	–0.56**	–0.60**	–0.24	–0.39*	–0.51**

*$P < 0.05$; **$P < 0.01$; [a] heavy metal in soil solution; [b] NH_4NO_3-extractable heavy metal; [c] total heavy metal; [1] lg(microbial biomass C); [2] lg(soil phosphatase activity).

organically bound and amorphous iron-manganese oxides Cu were the dominant fraction in the samples (n = 35), followed by the carbonates bound and residual Cu. In the case of Zn, the mean concentration of amorphous iron-manganese oxides Zn was the dominant fraction, followed by the organic-bound, carbonates, and residual fraction. The concentration of exchangeable Cu or Zn in the soil was very low and constituted only 0.39%–1.98% of the total Cu and 0.09%–0.66% of the total Zn, respectively (Fig.2).

2.4 Relationship of heavy metals and soil microbial biomass, enzyme activity

Microbial biomass carbon (C_{mic}), measured by fumigation-extraction in the soils ranged from 11.07 to 67.03 mg/kg (Fig.3). It increased significantly from the nearest plot (50 m) to the most distant plot (600 m). The plots situated within E7 contained nearly 4 times more C_{mic} than the most polluted soil (Fig.3). Soil lg C_{mic} was negatively correlated with soil solution Cu (r=–0.43), NH_4NO_3-extracted Cu (r=–0.60), total Cu (r=–0.64) and NH_4NO_3-extracted Zn (r=–0.38), total Zn (r=–0.48) (Table 3). The microbial quotient (C_{mic}/C_{org} ratio) varied widely and showed an increased trend with the decrease of heavy metal content (Fig.3 and Table 3). However, there was no significant correlation between microbial biomass C and soil organic C. Multiple regression analysis data using the NH_4NO_3-extractable heavy metals is as the follows:

$$\lg C_{mic} = 1.72 - 5.80 \times 10^{-4} C_{total\text{-}Cu} + 2.27 \times 10^{-2} C_{N\text{-}Zn} - 2.69 \times 10^{-5} C_{total\text{-}Zn} - 2.30 \times 10^{-2} C_{N\text{-}Cu} \qquad R^2 = 0.54 \quad (1)$$

where, $C_{N\text{-}Zn}$ or $C_{N\text{-}Cu}$ are NH_4NO_3-extractable Zn or Cu concentration.

We observed that 54% of the variation in lg C_{mic} could be explained by the NH_4NO_3-extractable and total heavy metal concentrations in the soils.

2.5 Enzyme activity

Variations of the alkaline phosphatase activity in the soil samples along the smelter are shown in Fig.4. A good relationship was observed between enzyme activity and the distance from the smelter. Phosphatase activities in soil samples were the lowest near the smelter. Soil phosphatase activity showed an increased trend with the decrease of

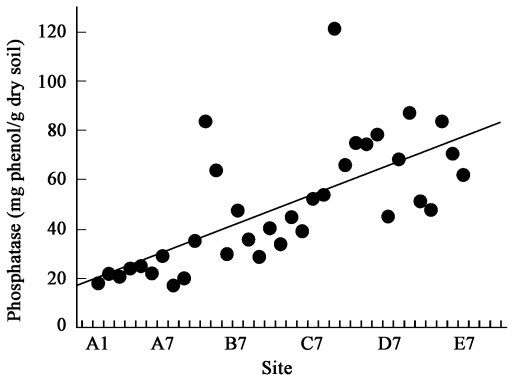

Fig. 4 Variations of phosphatase activities near a copper-zinc smelter.

heavy metal concentration. Soil lg (phosphatase activities) were negatively correlated with soil solution Cu (r=–0.47), NH_4NO_3-extractable Cu (r=–0.85), total Cu (r=–0.82), soil solution Zn (r=–0.68), NH_4NO_3-extractable Zn (r = –0.76) and total Zn (r=–0.61) (Table 3). 72% of the variability in phosphatase activity could be explained by the NH_4NO_3-extractable Cu according to the regression equation:

$$\lg P = 1.19 - 6.49 \times 10^{-2} C_{\text{N-Cu}} \qquad R^2 = 0.72 \qquad (2)$$

where, P presents soil phosphatase activity.

3 Discussion

All the regulations that define a maximum concentration in the receiving soil are based on total soil metal concentrations, which take no account of its availability in the soil. However, it is now generally accepted that plant uptake of metals depends on metal concentration in soil pore water and exchangeable heavy metals (Brun *et al*., 2001). Hence, it is the concentration of a metal in its available form that is crucial to metal toxicity. The solubility and fractions of heavy metals are usually related to plant uptake of heavy metals but effects of heavy metals on microbial activity are still poorly understood (Kunito *et al*., 1999; Sitaula *et al*., 1999; Huang and Shindob, 2000). Results of the present study indicate that heavy metals could reduce the activity of soil microorganisms (Table 3, Figs.3 and 4).

The concentrations of soil solution and exchangeable Cu, Zn in the soil were very low and constituted only 0.39%–1.98% of the total Cu and 0.09%–0.66% of the total Zn, respectively (Fig.1). The heavy metal concentrations in soil solution are generally reduced at neutral or alkaline pH (Munoz-Melendez *et al*., 2000). The study sites were found to be alkaline, where pH was as high as 8.27 (Table 2). These small but active fractions, however, may provide some indication of the forms of heavy metals that are most available soil microorganisms (Kunito *et al*., 1999; Vulkan *et al*., 2000; Gummuluru *et al*., 2002; Moffett *et al*., 2003).

Although the distribution of heavy metals among the various fractions determined by the sequential extraction scheme is not chemically clear-cut, the results of the fractionation provide an understanding of Cu or Zn relative to their potential mobility and bioavailability. We found that residual heavy metal contents in the soils were relatively low (Fig.2). Kabala and Singh (2001) also reported low percentages of residual copper in highly contaminated soils near a smelter in Poland. Possibly, the heavy metals in the soil underwent an accumulation process from readily available to unavailable fractions but hardly converted to the residual form.

The activity of soil microorganisms is closely related to soil fertility and environmental quality. Microbial biomass is considered as more sensitive indicator of changing soil conditions than total organic matter content. Brookes (1995) and McGrath *et al*. (2001) have provided evidence that heavy metal contamination decrease the proportion of microbial biomass C in total soil organic matter, and the ratio of soil microbial C to soil organic C has been proposed as a useful measure of soil pollution of heavy metals. Microbial physiological adaptation to heavy metals rely on several mechanisms, such as precipitation of metals as phosphates, carbonates, and sulphides, physical exclusion by exopolymers, and intracellular sequestration with low molecular weight cysteine-rich (Gadd, 1993; Yang *et al*., 2005). Such cellular mechanisms are energy-demanding, increase the maintenance energy and reduce the reduce the conversion of substrate into new microbial biomass and other metabolic processes. A reduction of this ratio as a result of metal pollution has been reported from other studies. $C_{\text{mic}}/C_{\text{org}}$ ratio increases mean a greater energy requirement for maintenance (Anderson and Domsch, 1989). Our results confirm these findings, because the $C_{\text{mic}}/C_{\text{org}}$ ratio values, expressing the maintenance energy, showed that more energy was consumed as the amount of Cu and Zn in soil increased (Table 3, Fig.3).

The above-mentioned hypothesis might also explain the reduction of enzyme activity observed in the Cu/Zn-contaminated soils. The results obtained for soil phosphatase indicate that the heavy metal contaminated soil along the smelter had significant measurable effects on the P-transforming activity of this enzyme (Table 3, Fig.4).

The availability of heavy metals influencing the soil microorganisms activity were investigated by a stepwise multiple regression analysis (Table 3). There was no significant influence of pH on the soil microorganisms. Phytoavailable Cu and Zn were found to significantly correlate with fulvic complex Cu and exchangeable Zn, respectively (Gummuluru *et al*., 2002). In the present study, the regression analysis of the data indicated that the toxicity of Cu and Zn to soil microorganisms was mainly through NH_4NO_3-extractable heavy metals. In addition, the soil in the experiments was multi-heavy-metal contamination. NH_4NO_3-extractable Cu and Zn together can be used to predict the toxicity of heavy metals to soil microorganism, which would explain the influence of combined pollution of Cu and Zn on soil microorganism activity.

4 Conclusions

Although the duration of our experiment does not allow prediction of microbial genetic diversity and potential

functional abilities, this will provide an explanation of the influence of combined pollution of Cu and Zn on soil microorganism activity along a heavy metals contamination gradient. The set of methods applied in this study was sensitive to site differences and contributed to a better understanding of heavy metals effects on activity of microorganism in soils. Subsequently, the results showed significant correlations between extractable contents of the heavy metals and soil microbial activity. Finally, we observed that 54% of the variation in $\lg C_{\mathrm{mic}}$, and 72% of the variation phosphatase activity could be explained by NH_4NO_3-extractable and total heavy metal concentrations.

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